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Abstract

Single-cell PCR and gene sequencing were conducted to evaluate species and genotypic compositions of *Cryptomonas* in the eutrophic Lake Hira, Japan. We determined the sequences of nuclear internal transcribed spacer 2 region from single *Cryptomonas* cells with a high success rate (83.3-97.9%), excluding one case (56.3%). A total of 325 sequences were obtained over 8 sampling days from May 28 to October 3 2012, and phylogenetic analysis indicated that all sequences were divided into six groups. Four groups were clustered together with known sequences of *C. curvata*, *C. marssonii*, *C. pyrenoidifera* or *C. tetrapyrenoidosa*, although the sequences of the other two groups did not show high similarity to known *Cryptomonas* species. *Cryptomonas curvata* dominated during the study period (45-98%), and unidentified *Cryptomonas* species (group 2) became dominant at high water temperatures. The genotypic composition of *C. curvata* also varied temporarily, suggesting that the genotypic composition of *Cryptomonas* was susceptible to environmental changes. These results indicated that single-cell PCR can be used to analyze the species composition and ecology of *Cryptomonas*.

Key words:

Cryptomonas, nuclear internal transcribed spacer 2 (ITS2), single-cell PCR, genotypic composition

Introduction

Microorganisms have four types of nutritional modes: heterotrophy, autotrophy, photoheterotrophy and mixotrophy. Based on specific nutritional strategies and evolutionary interests of mixotrophic algae, previous studies have explored their population, quantitative ecological role and life history traits in microbial communities (Porter 1988; Sanders and Porter 1988; Sanders 1991; Holen and Boraas, 1995). The genus *Cryptomonas* Ehrenberg (Ehrenberg 1831) is one of the most common mixotrophs that is ubiquitously distributed in lakes and ponds (Rott 1988; Menezes and Novarino 2003; Carino and Zingone 2006). Many ecological studies have been performed on *Cryptomonas* and have demonstrated that *Cryptomonas* plays an important role in aquatic ecosystems, such as periodical dominance (Graham et al. 2004), and acts as a grazer of bacterioplankton (Urabe et al. 2000) and prey of zooplankton (Porter 1973). However, the majority of studies have evaluated the dynamics of *Cryptomonas* populations at the genus level, and less information is available on species composition and ecological features of each species.

Classification criteria for *Cryptomonas* had been based on morphological features such as cell size, cell shape, furrow/gullet system, periplast structure, number of pyrenoids and internal anatomy (Novarino and Lucas 1993; Clay et al. 1999; Deane et al. 2002). However, previous molecular phylogenetic studies have clarified complex divergences in *Cryptomonas* and incongruence between morphological and phylogenetic classifications (Hoef-Emden and Melkonian 2003; Hoef-Emden 2005; Hoef-Emden 2007). Moreover, some *Cryptomonas* species show dimorphism (cryptomorph and campylomorph) or morphological plasticity and cannot be distinguished based on light microscopic observation alone (Hoef-Emden and Melkonian 2003; Hoef-Emden 2007). Therefore, molecular analysis

should be conducted to identify *Cryptomonas* species.

To evaluate the microorganism species composition, PCR-based molecular analyses, such as clone library analysis and denaturing gradient gel electrophoresis, have been applied. However, the quantitative accuracy of this data may be low since these results are biased by inhomogeneous amplification of PCR (von Wintzingerode et al. 1997). On the other hand, single-cell PCR methods without any bias for quantification of the microbial community have also been developed (Ki et al. 2005; Auinger et al. 2008; Bachvaroff et al. 2012). Nuclear internal transcribed spacer 2 region (ITS2) is variable and used for phylogenetic analysis and the identification of potential biological species for some lineages (e.g., Coleman 2000), though the plants and fungi have relatively conserved ITS2 regions (Caisová et al. 2013; Caisová and Melkonian 2014). The nuclear ITS2 region is also used for phylogenetic analysis of the genus *Cryptomonas* and reported that the high variabilities among the clades are identical within putative biological species (Hoef-Emden and Melkonian 2003). Although the ITS2 region seems not to be enough resolution for analyzing phylogenetic relationships among the species correctly (Hoef-Emden and Melkonian 2003; Hoef-Emden 2007), it has sufficient information to identify *Cryptomonas* at species level. Therefore, single-cell PCR targeting the nuclear ITS2 region seems to be the one of useful methods for evaluating species composition within the *Cryptomonas* community.

In the present study, we evaluated species/genotype compositions of *Cryptomonas* community in eutrophic Lake Hira, Japan, using a single-cell PCR method. We seasonally monitored nuclear ITS2 sequences from each *Cryptomonas* cell directly isolated from lake water and evaluated temporal variations in species compositions of *Cryptomonas*, as well as the genotypic compositions of each species

during the study period. These results, together with the biological and chemical factors that are thought to affect *Cryptomonas* populations, were used to explore the environmental factors that affect species and genotype compositions.

Materials and methods

Study site and sampling procedure

Lake Hira (35°2’ 57.12”N, 135°55’22.73”E) is a eutrophic satellite lake located on the eastern shore of Lake Biwa in Shiga prefecture, western Japan (Supplement a). The surface area and average depth are 0.134 km^2 and 1.5 m, respectively. Lake Hira connects to Lake Yanagihira via a small canal (Canal 1 in Supplement b) approximately 100 m long. The water from Lake Yanagihira flows into Lake Hira and finally into Lake Biwa through canal 2 (Supplement b). The water from Lake Biwa rarely flows into Lake Hira, even when the water level of Lake Biwa is higher than that of Lake Hira.

Samples from Lake Hira were collected semimonthly from 28 May 2012 to 19 November 2012. Because of the small area and the shallow depth, we supposed that Lake Hira is a typical continuous warm polymictic lake (Lewis 1983) and therefore collected the sample from the surface water. Surface water temperature was measured using a bar thermometer on each sampling day. Two liters of surface water were collected with a bucket and stored in a 4°C cooler box until the sample treatment.

Environmental factors

All treatments for chemical and biological analyses of the water samples were processed within 3 hours

after sample collection. Aliquots of water samples were filtered through pre-combusted Whatman GF/F filters (3 h at 420°C), and the filtrates were used to analyze dissolved inorganic nitrogen (DIN) and phosphorus (DIP). Nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}). These concentrations were measured using colorimetric analysis with an AACS II Auto Analyser (Bran+Luebbe, Norderstedt, Germany). The ammonium concentration was determined fluorometrically (Holms et al. 1999). For chlorophyll *a* (Chl. *a*) concentrations, seston in 10 ml of water were collected onto glass microfiber filters (Whatman® GF/F, GE Healthcare UK Ltd.) and stored at -30°C. Chlorophyll *a* concentrations were determined using the method of Welschmeyer (1994) with a spectrofluorophotometer (RF-5300, Shimadzu Corporation, Kyoto, Japan) after extraction with 10 mL of N,N dimethylformamide (Suzuki and Ishimaru 1990). We also measured daily precipitation at the Otsu station (Fig. 1) based on weather static information from the Japan Meteorological Agency (Japan Meteorological Agency 2013) and evaluated the effect of precipitation on the *Cryptomonas* community during the study period.

Estimation of Cryptomonas cell density

To estimate *Cryptomonas* cell density, 100 ml water samples were preserved in 1% glutaraldehyde. All *Cryptomonas*-like cells with orange fluorescence derived from phycobiliproteins under green excitation light were enumerated using a fluorescent microscope (BX50F4, OLYMPUS, Japan). At least 200 fields were counted on each slide at 400× magnification for three replications, and the density was calculated from the average cell number.

Single cell PCR and sequence analysis

We analyzed pigment-containing *Cryptomonas*-like (cryptomorph and campylomorph) cells, and did not evaluate colorless cells, including *C. paramaecium* (synonym: *Chilomonas paramaecium*). A total of 36 to 48 cells were isolated for each sample using a narrow-width glass capillary under a microscope, and cells were washed three to five times in sterilized water and put into 0.2-mL PCR tubes containing 20 µL of 10% Chelex® (Bio-Rad Laboratories, Hercules, California, USA) solution. The tubes were incubated at 95°C for 20 min. Since Chelex® solution inhibits PCR amplification, 10 µL of each supernatant was collected from extracted samples and used for template DNA of PCR amplification.

The ITS2 region of *Cryptomonas* is variable enough for species identification (Hoef-Emden and Melkonian 2003), and we designed a new primer set for obtaining the PCR product of the ITS2 region efficiently. To design a new primer set, a total of 31 *Cryptomonas* sequences were selected from Hoef-Emden and Melkonian (2003) and Hoef-Emden (2005) (Accession No., AJ566140, AJ 566142-566153, AJ566155-566157. AJ566159-566170, AJ715444 and AJ715454-715455, Fig. 2), and aligned using MEGA 5 (Tamura et al. 2011). The end region of 5.8S ribosomal DNA and the beginning region of 28S ribosomal DNA of all reference sequences were examined and the new primer set (Forward primer : 5'-GCACGCCTGTTTGAGGRA-3') and (Reverse primer : 5'-TGCTTAAGTTCAGCGGGTAG-3') was designed using Primer 3 Plus (Untergasser et al. 2007).

PCR amplification was performed with a 20-µL PCR mixture containing 1.0-µL template DNA from supernatant liquids of Chelex® extractions, 2 µL of ×10 Buffer for Blend Taq with 20 mM of Mg^{2+} (TOYOBO Co. Ltd., Osaka, Japan), 2 mM of each dNTP, 2.5 U Blend Taq-Plus- DNA polymerase

(TOYOBO Co. Ltd., Osaka, Japan) and 0.2 μL of each primer (0.2 mM). After pre-incubation at 94°C for 3 min, 35 cycles were performed at 94°C for 30 s, 56.6°C for 30 s and 72°C for 2 min, followed by a final extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) and sequenced using a Big Dye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems (Life Technologies Japan Ltd., Tokyo, Japan)) using an ABI3130xl Genetic Analyzer (Applied Biosystems (Life Technologies Japan Ltd., Tokyo, Japan)).

Alignment of ITS2 region and phylogenetic analysis

The obtained nuclear ITS2 alignments were folded using the mfold web server (Zuker 2003) for RNA secondary structure. The partial ITS2 region, mainly consisting helix domain III, was used in the unrooted phylogenetic analysis (cf, Hoef-Emden 2007). To infer helix domain III region in our obtained ITS2 sequences, we firstly made secondary structure prediction of the ITS2 region in all our sequences. We then compared our graphs with that of *C. obovoidea*, *C. erosa*, *C. commutata*, *C. loricata* and *C. phaseolu*s (Hoef-Emden 2007) and determined each helix III regions. Other known *Cryptomonas* species were referenced from Hoef-Emden (2007), DDBJ (DNA database of Japan) and BLAST search. The alignments were refined by eye and non-alignable regions were excluded prior to analysis. The 157 positions were used for the analysis.

Because ITS2 region is hyper variable and cannot be properly aligned among Cryptophyto sequences (Hoef-Emden 2007), the unrooted phylogenetic analysis was performed. For the unrooted phylogenetic analysis, neighbor-joining (NJ) and maximum likelihood (ML) analysis were performed

using MEGA 6.06 (Tamura et al 2013). In ML analysis, the selected optimum model was a Kimura 2 parameter (Kimura 1980) with gamma-distributed rates using a model selection option (a maximum likelihood value, lnL = -2402.4983, Tamura et al 2011). Thus we used this model for the unrooted phylogenetic analysis and set the bootstrap value at 2000. In NJ model, a bootstrap value was set at 2000. The topology of the consensus tree was the same as ML (data not shown), thus we only showed the result of the ML analysis.

Results

Variations in environmental factors in Lake Hira

Water temperature increased gradually from mid-June and reached a maximum of 34°C on August 16, and then decreased to 23°C by 3 October (Fig. 1a). Because the rainy season ranges from Jun to mid-July in Japan, the amount of daily precipitation varied during the study period, and high amounts of daily precipitation was observed more frequently during this period than other periods (Fig. 1a).

Nutrients were supplied mainly from inflow and increased during the rainy season (Fig. 1b). The DIN concentration peaked (180.8 μg L^{-1}) on 4 July and gradually decreased to less than 20 μg L^{-1} until 7 August (Fig. 1b). The concentration of DIP fluctuated from 28 May to 4 July and peaked (17.9 μg L^{-1}) on 19 July (Fig. 1b). The concentration gradually decreased and reached 0 μg L^{-1} on 25 September.

Variations in *Cryptomonas* density and species composition

The abundance of phytoplankton was affected by the high turnover rate of the lake water due to high

precipitation. The chlorophyll *a* concentration remained low (less than 10 μg L^{-1}) from mid-June to mid-July (Fig. 1c), although DIN and DIP were replete (Fig. 1b). Chlorophyll *a* concentration increased gradually from mid-July and reached 212.1 μg L^{-1} on 25 September.

Cryptomonas cell density increased from 4.3×10^3 cells mL^{-1} (28 May) to 4.1×10^4 cells mL^{-1} (4 June) during the first week, and then decreased to 2.0×10^3 cells mL^{-1} until 13 June (Fig. 1c). The density remained low and ranged from 1.4×10^3 cells mL^{-1} to 7.0×10^3 cells mL^{-1} after 4 June, although a minor increase and decrease in the density was observed four times (19 to 26 July, 7 August, 12 September and 3 October).

Single-cell PCR analysis was conducted eight times during the study period (Fig. 2). We successfully determined the sequences with high efficiency (83.3% -97.9%), excluding one date (56.3%, 7 August) (Fig. 2). A total of 325 sequences from 8 sampling days were obtained, and all sequences were clustered into six groups (Fig. 3). Four out of six groups were clustered together with known species and predicted to be *C. curvata* (group 1, 246 sequences), *C. tetrapyrenoidosa* (group 2, 30 sequences), *C. pyrenoidifera* (group 3, 5 sequences) or *C. marssonii* (group 4, 2 sequences). Groups 5 (23 sequences) and 6 (19 sequences) were phylogenetically located closest to *C. marsonii*, but did not include any known sequences (Fig. 3).

Group 1, which was closely related to *C. curvata*, dominated during the study period (44.4% to 97.7% of the total abundance), and the relative abundance tended to decrease during high water temperature periods (Fig. 2). Group 2, which was estimated to be *C. tetrapyrenoidosa*, also appeared throughout the period and accounted for 2.3% to 17.7% of the total abundance. Unidentified

Cryptomonas group 5 was detected from 4 June to 12 September, ranging from 4.9% to 15.2% of the total abundance. On the other hand, Group 3 (*C. tetrapyrenoidosa*) and Group 4 (*C. marsonii*) appeared sporadically and ranged from 0% to 5.0% and 0% to 6.1%, respectively. Unidentified *Cryptomonas* group 6 appeared and dominated only from 26 June (20.0%) to 7 August (40.7%).

Genotypic composition in *C. curvata*

Although we detected multiple genotypes for each species except for *C. marssonii* (group 4), enough number of sequence was obtained only for *C. curvata* (group 1) to analyze temporal variation in the genotypic composition (Fig. 4). A total of 36 genotypes in 246 sequences were presumed to belonging to *C. curvata* (group 1). Although most of the genotypes were observed with low frequencies during the study period (less than 8 sequences in total, see Fig. 4), the other 11 genotypes were detected repeatedly and can therefore be categorized into the following four groups; the first group was present during the entire study period and seemed to decrease at high water temperatures (C1, C3, C10, C11 and C16), the second and third groups appeared only in the early or late parts of the study period (C8, C9 and C13), and the fourth group appeared and dominated from 26 June to 7 August (C15 and C17).

Discussion

Due to the difficulty in the morphological identification of *Cryptomonas*, most of the studies on the phytoplankton community in natural systems showed *Cryptomonas* by genus level abundance. The result of this is that information about the species composition and its seasonal variations are quite limited.

Moreover, morphological classification does not work for some *Cryptomonas* species (Hoef-Emden and Melkonian 2003; Hoef-Emden 2007). Using single-cell PCR, we could analyzed the species composition and its variation even for morphologically indistinguishable species *C. curvata* and *C. pyrenoidifera* (Fig. 3). Therefore, this approach seems to be an effective method for evaluations of the species composition and diversity in natural phytoplankton communities. On the other hand, single-cell PCR approach largely lacks the morphological information of target cells, since we extract DNA from living cells without the fixation and following microscopic observation. This does not affect its availability for the identification and analysis of the species diversity, but becomes problematic for studies examining the life history of individual species especially those showing dimorphism. Further change or revision of the method may be needed to study the evolution of morphological plasticity.

At least 14 *Cryptomonas* species have been recognized based on molecular analyses (Hoef-Emden 2007). In Japan, six species (*C. erosa*, *C. ovata*, *C. platyuris*, *C. rostratiformis*, *C. tetrapyrenoidosa* and *C. paramecium*) have been recorded in previous studies (Mizuno 1971; Ishimitsu and Chihara 1984; Erata and Chihara 1987; Alam et al. 2001), whilst *C. curvata*, *C. pyrenoidifera* and *C. marssonii* were firstly observed in the present study. On the other hand, these three species do not seem to be rare and have been frequently reported in Asia such as China and Korea (e.g., Kim et al 2007; Choi et al. 2013; Xia et al. 2013). Because of the difficulty of morphological identification and recent revisions of *Cryptomonas* species, *Cryptomonas* species observed in Japan have not been fully described, and some species have been identified as different species. For example, *C. platyuris* NIES-276 isolated in Japan was morphologically re-identified to *C. borealis* (Hoef-Emden and Melkonian 2003). *Cryptomonas*

rostratiformis seems to be one of the frequently observed species in Japan (e.g., Ishimitsu and Chihara 1984; Erata and Chihara 1987). However, the species was emended and became a synonym of *C. curvata*, because the strain M1484 from Germany being re-identified to the campylomorph strain of *C. curvata* both from morphological and molecular phylogenetic perspective (Hoef-Emden and Melkonian 2003). Therefore, the *C. rostratiformis* reported and isolated in Japan should be reevaluated in light of the current classification. Moreover, *C. curvata* and *C. pyrenoidifera* show dimorphism and exhibit similar cryptomorphs (Hoef-Emden and Melkonian 2003), which may also account for the species disregard in Japan.

In pelagic region of typical dimictic lakes with large water surface area, seasonal variations in phytoplankton abundance of the surface water are relatively predictable and mainly affected by water temperature and nutrient supply in the water column by seasonal vertical mixing. On the other hand, water retention time or water exchange rate become more important factors in water systems with highly susceptible to variable influent quantities such as small lakes and ponds, upstreams of river-mouth dams, and riverine and transitional zones of reservoirs (e.g., Thornton et al. 1990; Hodoki and Murakami 2007; Yamamoto et al. 2013). Lake Hira is small and shallow, and previous study also showed that nutrients (DIP and DIN) are usually remaining in the water (Mitamura et al. 2014) and seem not to become limiting factors for phytoplankton growth. Therefore, the frequent lake water exchange due to precipitation seemed to affect *Cryptomonas* density and total phytoplankton abundance, and kept their levels low from early June to mid-July (Fig. 1b). On the other hand, biomass in phytoplankton increased after rainy periods, and nutrient depletion gradually became a limiting factor for phytoplankton growth rate during

the study period (Fig. 1c).

Because of the limitation of morphological classification, we should pay attention to interpretations of the ecological features of *Cryptomonas* morphologically analyzed in previous studies. In Lake Hira, *C. curvata* was the dominant species throughout the study period (Fig. 2). This species has been reported worldwide (e.g., Menezes and Novarino 2003; Melo et al. 2004; Xia et al. 2013). Seasonal changes of the *Cryptomonas* species composition in a Brazilian reservoir indicated that *C. curvata* tended to dominate during low temperature periods (15-20°C, between March and September), and then decreased during the summer (Bicudo et al. 2009). The relative abundance of *C. curvata* in L. Hira also remained low during the high water temperature period (> 30°C), and our results were consistent with previous reports (Fig. 2).

Unidentified *Cryptomonas* groups phylogenetically similar to *C. marssonii* also appeared and became dominant during high water temperature periods (Fig. 2). *Cryptomonas marssonii* is ubiquitously distributed under various environmental conditions (Smolander and Arvola 1988; Kim et al. 2007). In Brazil, *C. marssonii* is one of the most common *Cryptomonas* species (Menezes and Novarino 2003), and its density increases during high temperature periods (Bicudo et al. 2009). Rott (1988) examined flagellate assemblages in four mid-altitude lakes (average surface water temperature of 24.3˚C) and four high-mountain lakes (average surface water temperature of 10.8˚C) in Austria, and reported that *C. marssonii* was more abundant in mid-altitude lakes. In the present study, the response of the unidentified groups to water temperature was consistent with previous studies showing higher abundance at high water temperatures, and environmental characteristics of these groups resembled *C. marsonii*.

Genotypic diversity and its role have been investigated for cyanobacteria. For example,

high-light-adapted genotypes of *Prochlorococcus* were phylogenetically classified into the same clade (Moore et al. 1998). *Cuspidothrix issatschenkoi* consists of at least three ecotypes due to the various combinations of toxin production and nitrogen-fixation abilities, and nitrogen-fixation-lacking and toxin-producing genotypes prefer more nitrogen-replete environments (Hodoki et al. 2013). Genotypic composition of *Microcystis aeruginosa* varies temporally and locally depending on the environmental condition, although all genotypes are distributed ubiquitously (Briand et al. 2009; Sabart et al. 2009; Ohbayashi et al. 2013). In the present study, the genotypic composition of *C. curvata* also varied temporary during the study period (Fig. 4). However, we analyzed only ITS 2 regions, and the sequence information is not enough for speculating the phylogenetic relationships among the genotypes correctly. Therefore, we could not discuss the response of each genotype to environmental factors, and then the reason of variations in genotypic composition of *C. curvata* observed in the present study was unclear. To clarify this problem, further ecophysiological studies are required with increasing the sequence information analyzed by single-cell PCR.

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3

4

Figure captions

Supplement. Location of Lake Hira (a) and the study site in Lake Hira (b)

Fig. 1 Changes in water temperature and the amount of daily precipitation (a), dissolved inorganic nitrogen (DIN) concentration and dissolved inorganic phosphorus (DIP) concentration (b), and chlorophyll *a* concentration and *Cryptomonas* density (c)

Fig. 2 Relative species composition of the *Cryptomonas* community in Lake Hira. Numbers on the bar indicate numbers of analyzed sequences / isolated cells

Fig. 3 Unrooted maximum likelihood tree based on ITS2 Helix III regions (157 positions). Boostrap values >50% are indicated near the node. Numbers in parentheses indicated near the same sequences observed during the study period. Our 18 sequences similar to C. curvata were omitted from the phlogenetic analysis, since they were detected only once during study period.

Fig. 4 Temporal variation in the genotypic composition of *C. curvata* during the study period. Each number in parentheses represents the number of sequences obtained from each sampling day. Only genotypes observed more than eight sequences were used for analysis.

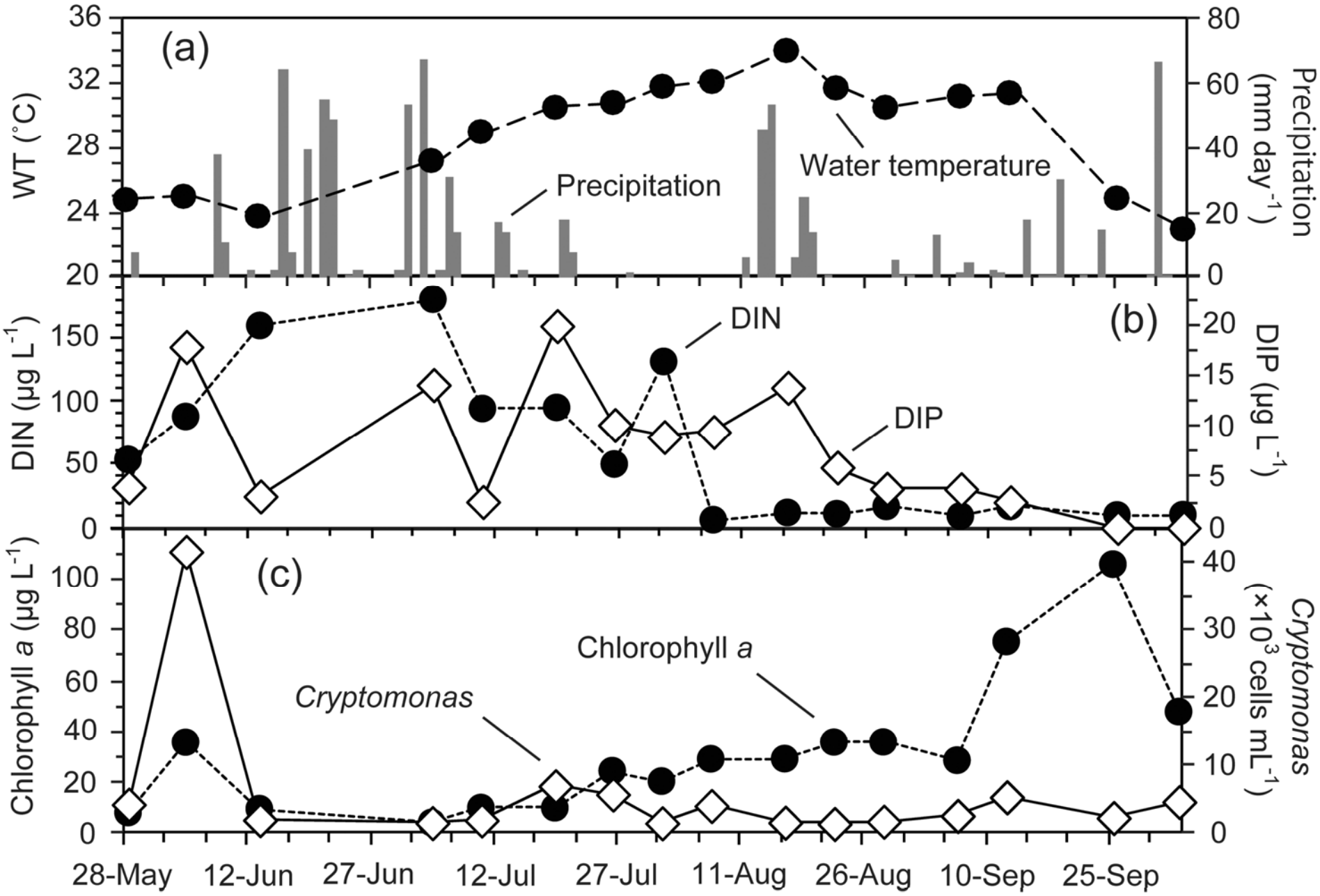
(a)
WT (°C)
Precipitation (mm day^{-1})
Water temperature
Precipitation
(b)
DIN (µg L^{-1})
DIP (µg L^{-1})
DIN
DIP
(c)
Chlorophyll *a* (µg L^{-1})
Cryptomonas (×10^3 cells mL^{-1})
Chlorophyll *a*
Cryptomonas
28-May
12-Jun
27-Jun
12-Jul
27-Jul
11-Aug
26-Aug
10-Sep
25-Sep

Fig. 1

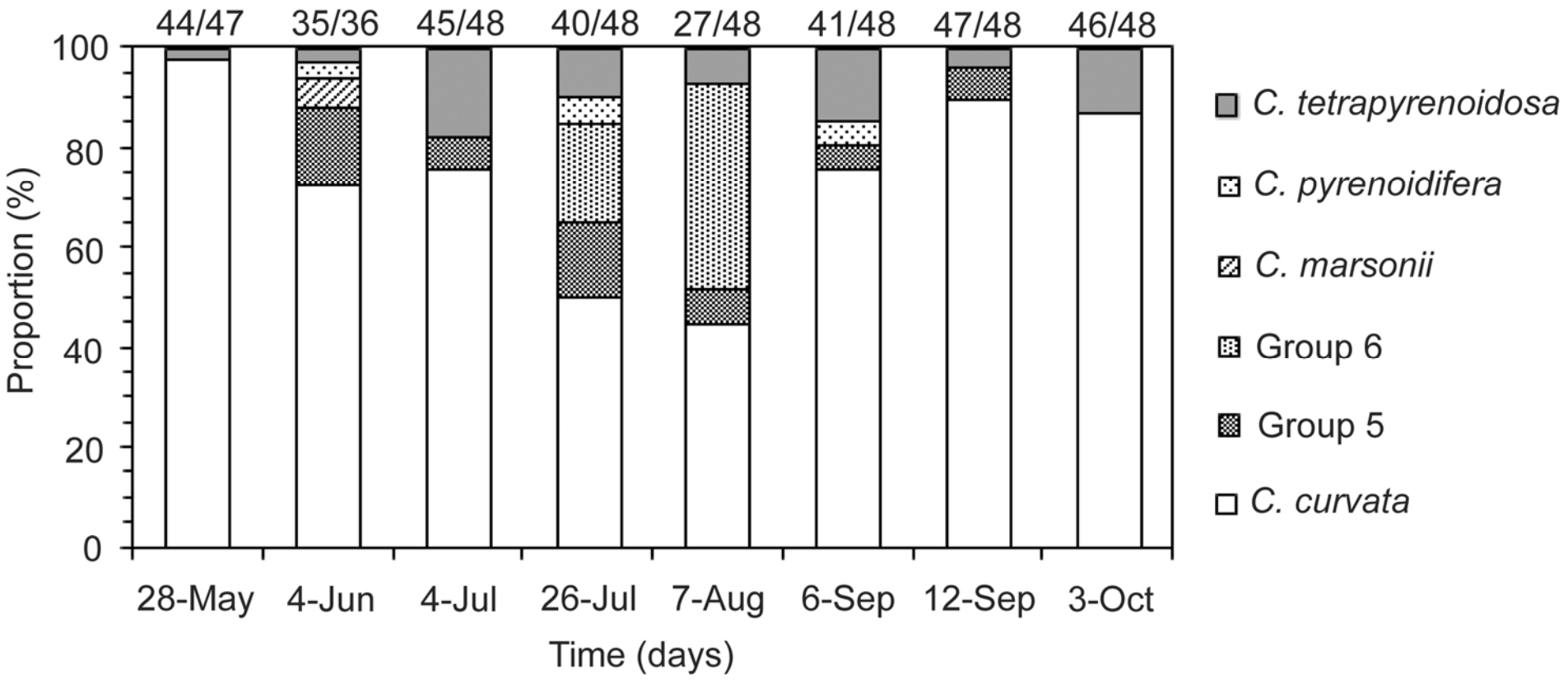
44/47
35/36
45/48
40/48
27/48
41/48
47/48
46/48
100
80
60
40
20
0
Proportion (%)
28-May
4-Jun
4-Jul
26-Jul
7-Aug
6-Sep
12-Sep
3-Oct
Time (days)
C. tetrapyrenoidosa
C. pyrenoidifera
C. marsonii
Group 6
Group 5
C. curvata

Fig. 2

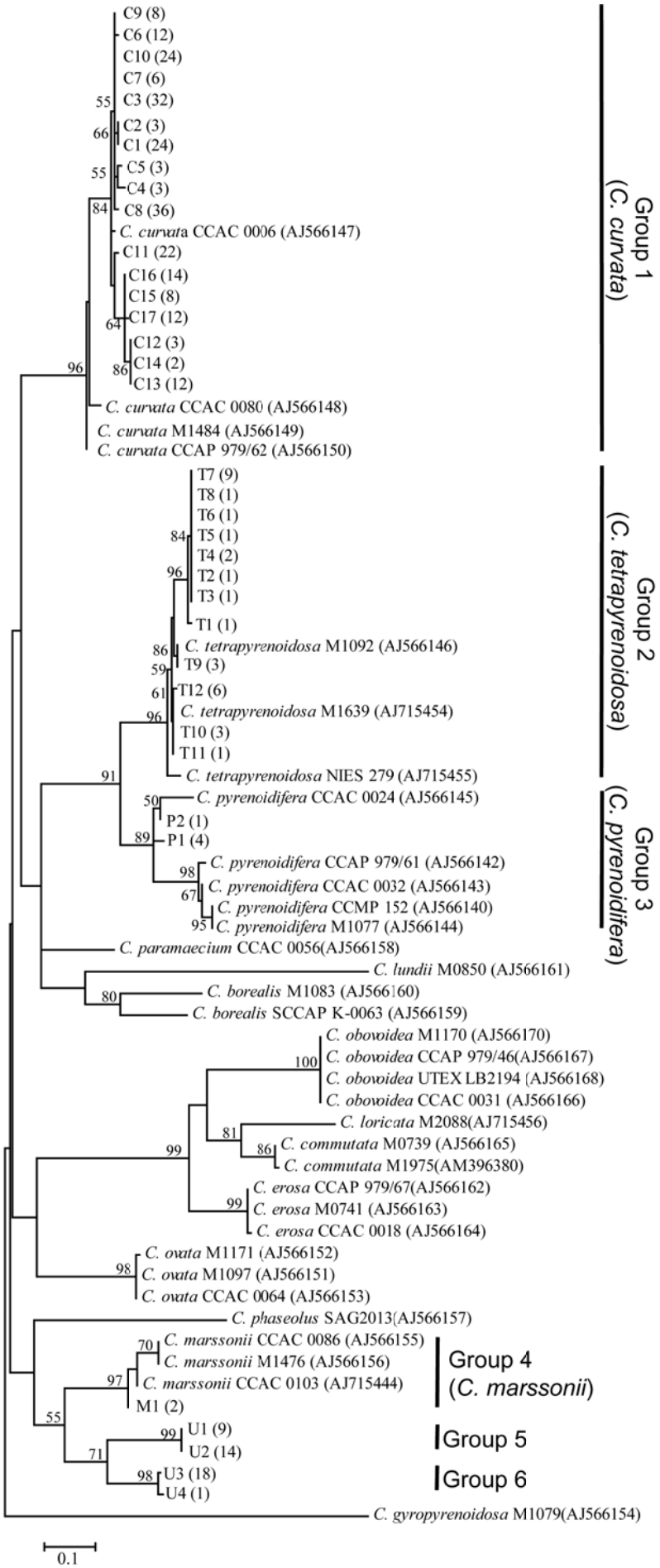

Fig. 3

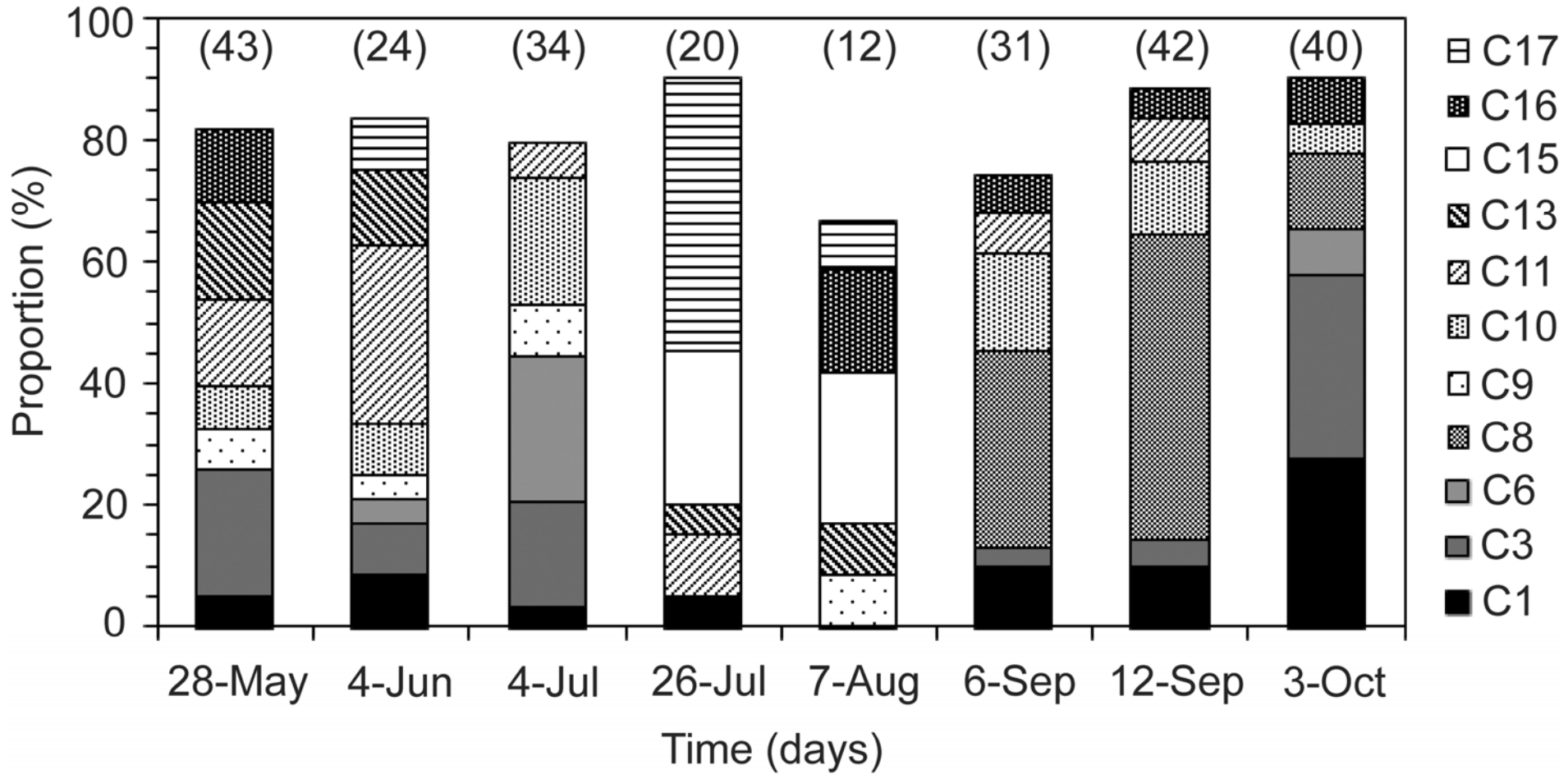
100
80
60
40
20
0
Proportion (%)
(43)
(24)
(34)
(20)
(12)
(31)
(42)
(40)
28-May
4-Jun
4-Jul
26-Jul
7-Aug
6-Sep
12-Sep
3-Oct
Time (days)
C17
C16
C15
C13
C11
C10
C9
C8
C6
C3
C1

Fig. 4